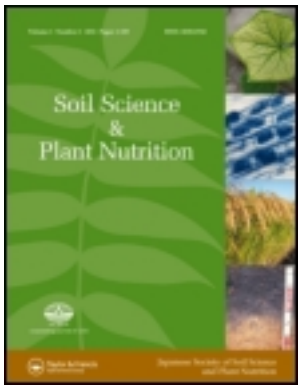

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Effect of Application of Rice Straw and Compost on the Bacterial Communities Associated with Moina sp. in the Floodwater of a Paddy Soil Microcosm: Estimation Based on DGGE Pattern and Sequence Analyses

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Effect of Application of Rice Straw and Compost on the Bacterial Communities Associated with *Moina* sp. in the Floodwater of a Paddy Soil Microcosm: Estimation Based on DGGE Pattern and Sequence Analyses

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Bacterial communities associated with *Moina* sp. in the floodwater of a paddy field microcosm were examined by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA. Eighteen out of 20 eubacterial DGGE bands were sequenced. The associated eubacterial communities mainly consisted of the *Cytophaga-Flavobacterium-Bacteroides* group and α-, β-, and γ-Proteobacterial groups, irrespective of the application of rice straw and rice straw compost. The effect of the application of rice straw and compost on the communities was not appreciable, compared with host specificity. An uncultured Cytophagales bacterium was estimated to be specifically associated with *Moina* sp. Presence of bacteria that are specific to rice straw treatment was also estimated.

Key Words: **bacterial community, DGGE, *Moina*, rice straw, 16S rDNA.**

Close and specific associations of microcrustaceans with microorganisms and algae are common in aquatic habitats. *Vibrio* spp. were the most commonly observed bacterial epibionts of marine copepods (Kaneko and Colwell 1975; Sochard et al. 1979; Huq et al. 1983; Carli et al. 1993; Hansen and Bech 1996; Carman and Dobbs 1997; Montanari et al. 1999). *Pseudomonas, Cytophaga, Acinetobacter* and *Flavobacterium* have also been observed as copepod epibionts in marine and freshwater environments (Sochard et al. 1979; Holland and Hergenrader 1981; Ho and Perkins 1985; Hansen and Bech 1996; Carman and Dobbs 1997). Bacterial communities associated with microcrustaceans are influenced by their hosts. Niswati et al. (2005) compared bacterial communities associated with five microcrustaceans (*Tanycypris* sp., *Moina* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp.) from the floodwater of a paddy field microcosm using molecular methods, and observed the presence of host-dependent bacterial communities.

Cladoceran *Moina* is an aquatic microcrustacean that occurs abundantly in the floodwater of paddy fields (Ali 1990; Ferrari et al. 1991; Kuwabara 1999; Yamazaki et al. 2001; Niswati et al. 2002). It plays an important role in the food web and in nutrient cycling in the floodwater of paddy fields due to the grazing of algae and bacteria (Wilson et al. 1980; Grant et al. 1983) and regulation of the bacterial communities in aquatic environments (Jürgens 1994).

Application of organic materials such as rice straw and compost, which is a common practice in rice cultivation, affected the community structure of the free-living bacteria in the floodwater of paddy fields (Okabe et al. 2000; Kimura et al. 2002). Niswati et al. (2003) also reported some effects of rice straw and compost application on the bacterial communities associated with *Moina* sp. and *Cypretta* sp. by restriction fragment length polymorphism (RFLP) analysis. The frequency of appearance of *Moina* sp. in the floodwater differed depending on the kind of fertilizers applied (Yamazaki et al. 2001). These findings indicate the development of phylogenetically specific bacteria in association with respective microcrustaceans in the floodwater of paddy fields, depending on the kind of fertilizers applied.

The purpose of the present study was to analyze the effect of application of rice straw and compost on eubacteria associated with *Moina* sp. phylogenetically, by using PCR-DGGE and subsequent sequencing analyses.

Materials and methods

This microcosm experiment was the same as that conducted by Niswati et al. (2003) for evaluating the effect

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of the application of rice straw and compost on the PCR-RFLP patterns of the eubacterial communities associated with five microcrustaceans. The DNA extracted from the *Moina* samples in the previous experiment was used in the present study.

Preparation of *Moina* sp. Individuals of *Moina* sp. were collected on May 7, 2001 from the floodwater of a paddy field located at Aichi-ken Anjo Research and Extension Center, Central Japan (latitude 34°48′N, longitude 137°30′E). They were cultured in an aqueous medium containing 5 g L^{-1} of chicken manure (N: 2.58%, P_2O_5: 8.11%, K_2O: 3.37%, CaO: 19.21%) and 0.1 g L^{-1} of baker yeast with periodical supply of *Chlorella* sp.

Paddy field microcosm. Soil samples used in the microcosm experiment were taken from the paddy field previously mentioned (Anthraquic Yellow Soil; Oxiaquic Dystrochrept). Properties of the soil samples were as follows: total C content, 13.3 g kg^{-1}; total N content, 0.9 g kg^{-1}; pH(H_2O), 6.0; pH(KCl), 4.9. One kilogram of soil that was passed through a 4-mm sieve was mixed thoroughly with chemical fertilizers consisting of $(NH_4)_2SO_4$, $Ca(H_2PO_4)_2 \cdot H_2O$ and KCl at the rates of 0.5, 0.5 and 0.2 g kg^{-1}, respectively (NPK treatment). Rice straw (about 2 cm long) or rice straw compost was mixed thoroughly into the fertilized soil at the rate of 10 g kg^{-1} on a dry weight basis (NPK+rice straw and NPK+compost, respectively). To avoid the emergence of indigenous microcrustaceans other than inoculated *Moina* sp., the soil samples were heated at 80°C for 2 h for partial sterilization. The soil samples were put into a container (20 cm long, 13 cm wide, and 14 cm high) and were submerged with 3 L of distilled water. Each treatment was prepared in duplicate.

Incubation and sampling of *Moina* sp. About 150 individuals of adult *Moina* sp. were inoculated to the paddy field microcosm 1 d after the set-up. The containers were placed in a plant growth chamber at 30°C (daytime) and 20°C (night time). During the incubation period, the depth of water was maintained at 10 cm with distilled water.

One hundred individuals of adult *Moina* sp. were sampled weekly. They were washed at least three times in sterile distilled water and were stored in a 1.5 mL tube with 1 mL of sterile ultra-pure water at −20°C until DNA extraction.

DNA extraction, PCR, and DGGE. DNA was extracted from the microcrustacean samples using a high temperature, salt and sodium dodecyl sulfate (SDS)-based lysis method (Zhou et al. 1996) with slight modifications for the extraction of a small volume of *Moina* sp. samples (Niswati et al. 2002). The DNA extracts were stored at 4°C for immediate use or at −20°C for long storage.

Eubacterial 16S rDNA was amplified by PCR with the 357f-GC and 517r primer pair (Muyzer et al. 1993). Conditions for PCR and DGGE were the same as those used for analyzing the eubacterial communities associated with five microcrustaceans reported by Niswati et al. (2005).

Sequencing of excised DGGE bands. The DGGE bands were carefully excised under UV illumination and placed in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted from the gel piece by over-night incubation at 4°C, and the supernatant was used as template DNA in the reamplification by PCR, with the same primer pair (357f and 517r) as that described above. The distinctly separated bands were directly sequenced. Sequence reactions were performed using a Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, NJ, USA), according to the manufacturer's instructions. The PCR products were analyzed with an automatic sequencer (model 373S, Applied Biosystems, CA, USA). When direct sequencing failed, the amplified DNA of the excised DGGE bands was cloned into the pT7Blue T-vector (Novagen, Darmstadt, Germany). The plasmids were transformed into competent cells of *E. coli* XL I-blue (Toyobo, Osaka, Japan). Plasmid DNA was extracted from the clones with a QIAprep spin miniprep kit (Qiagen, Crawley, UK). The clones with correct inserts were sequenced as described above.

Phylogenetic analysis of characteristic DGGE bands. Sequences were compared with the sequence information available in the GenBank database. The BLAST search option of the DNA Data Bank of Japan (http//:www.ddbj.nig.ac.jp) and the National Center for Biotechnology Information (http//:www.ncbi.nlm.nih.gov) was used to determine the closest relatives corresponding to the 16S sequences (Altschul et al. 1997). The sequences obtained in this study are available of DDBJ under accession numbers from AB115922 to AB115935 and from AB115937 to AB115943.

Statistical analysis of DGGE patterns. The observed DGGE band patterns were statistically analyzed based on their mobilities and intensities (Niswati et al. 2005). Cluster analysis was performed using the BlackBox program, according to the Ward method (Aoki 1996). Principal component analysis was performed using the Sristat program in EXCEL STATISTICS 97 for Windows (SRI, Tokyo). Correlation matrix was used in this analysis.

Results and discussion

Eubacterial communities

As shown in Fig. 1, the application of organic materials resulted in DGGE patterns different from that of the NPK treatment. Each pattern was reproducible between

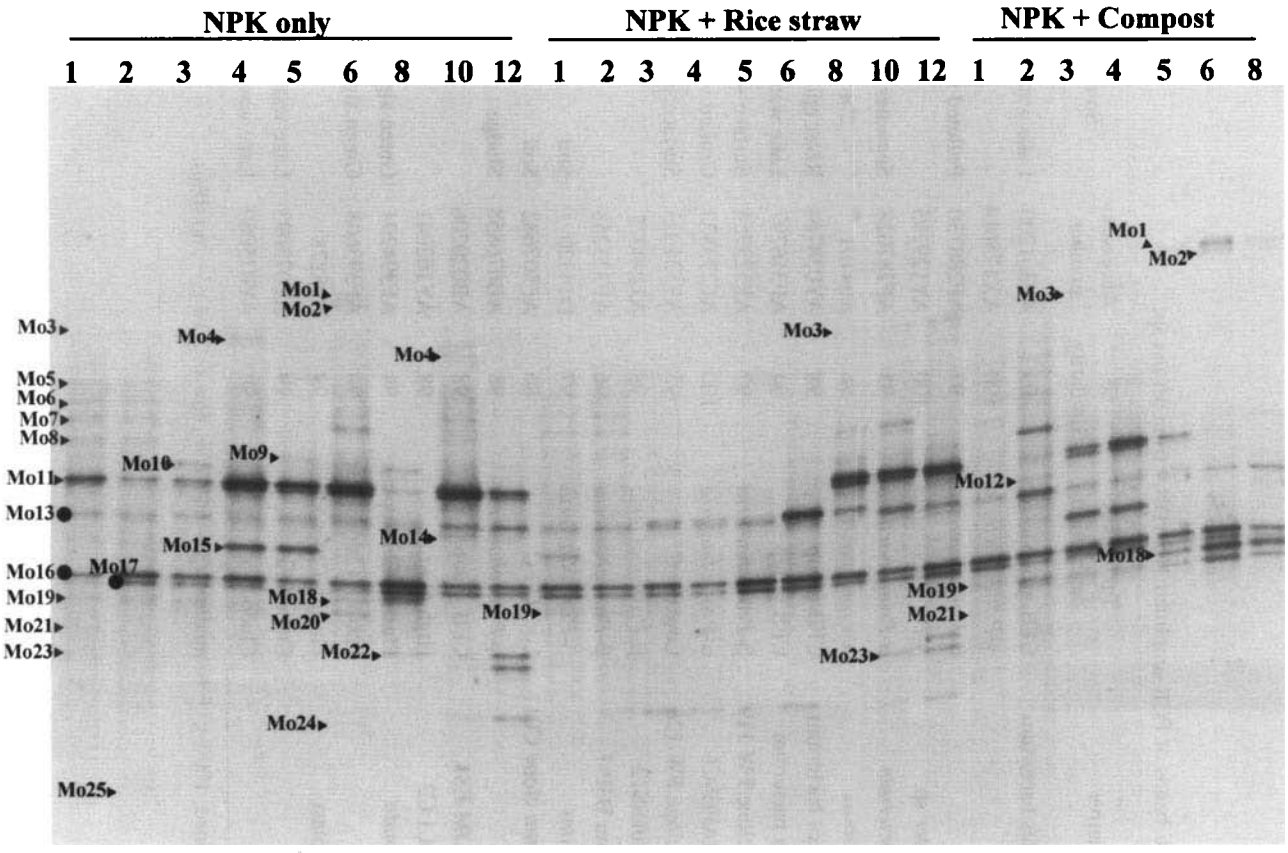

Fig. 1. Negative images of DGGE band patterns of the eubacterial communities during different incubation periods under different soil treatments. Soil treatment and incubation period (week) are indicated above the respective lanes. ●, common bands; ▶, bands specific to respective treatments and sampling times.

duplicates (data not shown). The total number of bands with different mobilities was 25 for all three treatments, among which 24, 15 and 20 different bands were observed in the NPK, NPK+rice straw and NPK+compost treatments, respectively. Three bands (Mo13, Mo16 and Mo17) appeared constantly or with a high frequency, irrespective of the application of organic materials and incubation period (Table 1). The frequency of appearance was calculated from the detection frequency of the respective bands in the total number of samplings (7 or 9 samplings). Although the frequency of appearance was different among the treatments, thirteen DGGE bands were commonly present in every treatment (Table 1). The abundance of the DGGE bands along with the duration of incubation is shown in Fig. 2. Although the number of DGGE bands in the NPK+rice straw treatment was lower in the middle incubation period than in the other treatments, there was no statistical difference in the mean number of bands among the treatments (9.9±2.6, 7.8±2.4 and 9.3±1.7 for the NPK, NPK+rice straw and NPK+compost treatments, respectively). The findings of the constant presence of three bands and the large number of bands commonly observed in every treatment suggested the development of specific eubacterial communities associated with *Moina* sp., irrespective of the kind of fertilizers, which was also observed in the PCR-RFLP analysis (Niswati et al. 2003).

Sequencing of DGGE bands

Among the 25 DGGE bands with different mobilities, 23 bands were successfully sequenced. Closest relatives corresponding to the respective bands are listed in Table 1. Many of the closest relatives belonged to Proteobacteria or to the CFB group. The closest relative corresponding to band Mo13 was an uncultured Cytophagales bacterium. As this band appeared on every sampling occasion, irrespective of the treatment (Table 1), and was absent in the bacterial communities associated with *Tanycypris* sp., *Cypretta* sp., *Heterocypris* sp. and *Mesocyclops* sp. (Niswati et al. 2005), a Cytophagales bacterium with the same sequence as that of band Mo13 was considered to be the bacterium specifically associated with *Moina* sp. CFB members are known to be abundant in both freshwater and marine environments (Kirchman 2002) and to form a symbiosis and/or an association with invertebrate hosts such as a terrestrial crustacean *Porcellio scaber*, a wasp *Encarsia pergandiella*, a cockroach *Periplaneta america*, an acanthamoebae and a

Table 1. Closest relatives corresponding to the sequenced DGGE bands of PCR-amplified 16S rDNA from *Moina* sp.

Band name[a]	Soil treatment			Seq. (bp)	Alignment	Closest relative	Taxonomic description	% Similarity	Accession number	Sources
	NPK only	NPK+RS[b]	NPK+Cmp[c]							
Mo13	+++[d]	+++	+++	154	153/154	Uncultured Cytophagales bacterium	CFB group	99	AF361200	Lake water
Mo5	++	++	++	160	160/160	*Pseudomonas* sp.	γ-*Proteobacteria*	100	AX175614	
Mo7	+	+	++	160	156/160	*Variovorax paradoxus*	β-*Proteobacteria*	97	AF288730	Polluted soil
Mo10	+	++	++	129	129/129	Uncultured *Acinetobacter* sp.	γ-*Proteobacteria*	100	AY129795	
Mo19	++	+	+++	135	129/135	Uncultured α-*Proteobacterium*	α-*Proteobacteria*	95	AF287028	Seawater
Mo23	++	+	+	136	132/134	*Sphingomonas paucimobilis*	α-*Proteobacteria*	98	X94101	Contaminated soil
Mo8	+	++	+	155	153/155	Uncultured Bacteroidetes bacterium	CFB group	98	AY038764	River epilithon
Mo3	++	+	+	135	93/98	Uncultured Crater Lake bacterium	CFB group	94	AF316797	Lake water
Mo15	+	+	+	157	97/99	Uncultured bacterium clone FW 119	β-*Proteobacteria*	97	AF523954	Surface sediments
Mo24	+	+++	−	135	135/135	Uncultured bacterium IAFESC6	α-*Proteobacteria*	100	AF273323	Ground water
Mo1	+	−	++	138	92/96	Uncultured bacterium clone FW 128	Low G+C	95	AF524023	Surface sediments
Mo18	+	−	++	144	142/144	Uncultured bacterium fukuS12	β-*Proteobacteria*	98	AJ290075	
Mo21	+	−	+	162	150/155	Gram negative bacterium B3P-1	β-*Proteobacteria*	96	AF323258	
Mo22	+	+	−	160	156/160	*Brachymonas denitrificans*	β-*Proteobacteria*	97	D14320	Soil
Mo2	+	−	+	138	89/103	Uncultured soil bacterium clone C011	Low G+C	86	AF507682	Soil
Mo4	+	−	−	160	157/160	*Aeromonas* sp. Pg-3	γ-*Proteobacteria*	98	AB076858	Sludge
Mo14	+	−	−	155	153/155	*Sphingobacterium* sp. OM-E81	CFB group	98	AB020206	
Mo25	+	−	−	139	129/131	Actinobacterium PI GH.1.C2	High G+C	98	AY162041	
Mo11	+++	+	++	138	130/138	*Chlamydomonas applanata*	Plastid, Chloroplast	94	AF394204	Green algae
Mo6	++	++	+	137	125/130	*Pyramimonas parkeae*	Plastid, Chloroplast	96	AF393608	Green algae
Mo9	+	−	+	137	136/137	*Alnus glutinosa* chloroplast	Plastid, Chloroplast	99	X95278	
Mo16, Mo17	+++	+++	+++	136	135/136	*Bosmina coregoni*	Cladocera	99	AY075093	Lake water
				136	135/136	*Polyphemus pediculus*	Cladocera	99	AY075080	Lake water

[a]See Fig. 1. [b]RS, rice straw; [c]Cmp, compost. [d]Appearance frequency: +++, high (>66%; underlined, 100%); ++, medium (33–66%); +, low (<33%); −, no (0%).

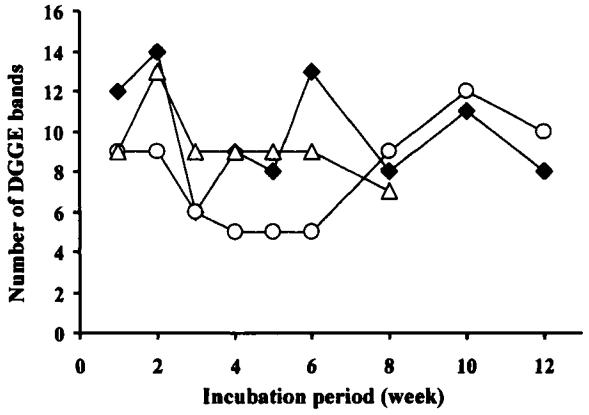

Fig. 2. Temporal changes in the number of DGGE bands under different soil treatments. ◆, NPK only; ○, NPK+rice straw; △, NPK+compost treatment.

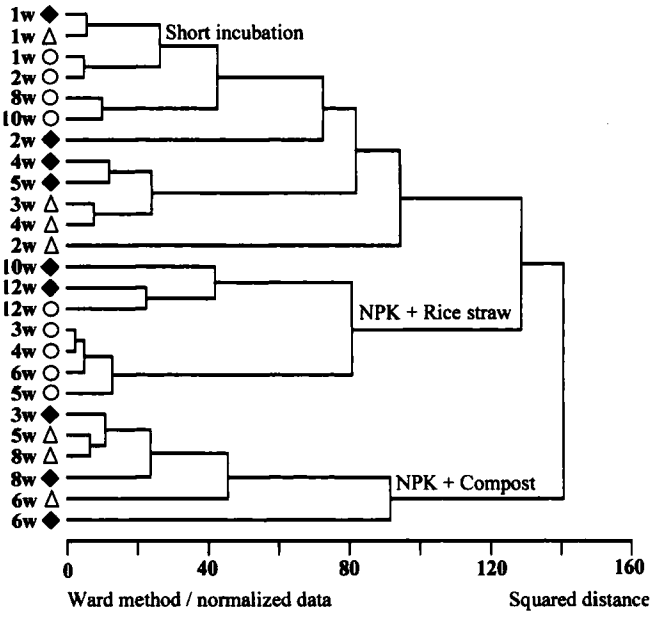

Fig. 3. Cluster analysis of DGGE band patterns of eubacterial communities under different soil treatments. Digital numbers before the letter w indicate the duration of incubation after flooding (week). ◆, NPK only; ○, NPK+rice straw; △, NPK+compost treatment.

shrimp *Neotrypaea californiensis* (Dugas et al. 2001; Horn et al. 2001; Zchori-Fein et al. 2001; Kostanjšek et al. 2002; Lau et al. 2002).

In spite of the low intensity, band Mo5 (closest relative corresponding to *Pseudomonas* sp.) was common in all the treatments with a medium frequency of appearance (Table 1). In contrast, bands Mo24 and Mo19 appeared with a high frequency in the NPK+rice straw treatment and NPK+compost treatment, respectively. As bands Mo5, Mo10 and Mo19 that were detected in every treatment were absent in the bacterial communities associated with other microcrustaceans in the previous study (Table 1; Niswati et al. 2005), the eubacteria corresponding to these bands were considered to be specifically associated with *Moina* sp., irrespective of the treatment. In contrast, as band Mo24 was observed in the bacterial communities of all the microcrustaceans under rice straw application (Niswati et al. 2005), it was considered to be specific to the NPK+rice straw treatment.

Bands Mo1, Mo9, Mo18, Mo21 and Mo2 did not appear in the NPK+rice straw treatment, while bands Mo24 and Mo22 did not appear in the NPK+compost treatment (Fig.1, Table 1). Bands Mo7 and Mo8 were detected in the bacterial communities of *Tanycypris* sp., while bands Mo3 and Mo15 were observed in the bacterial communities of many host microcrustaceans (Niswati et al. 2005).

Bands Mo16 and 17 that were also constantly detected were estimated to be derived from the 16S rDNA of *Moina* sp. itself. In addition, bands Mo6, Mo9 and Mo11 were considered to be derived from plastids of eukaryotes (Table 1). Chang and Jenkins (2000) also isolated the plastid of eukaryotic algae from *Daphnia obtusa*, which indicated the close association of eukaryotic algae with *Moina* sp. Based on the 18S rDNA analysis of associated eukaryotes with *Moina* sp., the amplified plastids in the present experiment were estimated to be derived from a Volvocales alga (data not shown).

Effect of organic matter application on eubacterial communities

As shown in Fig. 3, cluster analysis of the DGGE band patterns tended to separate the bacterial communities into three clusters: 1) early period of incubation, 2) NPK+rice straw treatment, and 3) NPK+compost treatment. Bacterial communities in the NPK treatment were distributed in every cluster. Physicochemical conditions and the aging of the *Moina* sp. appeared to induce changes in the band patterns along the duration of incubation. Since Pérez-Martínez and Gulati (1999) reported that the amount of nitrogen released to the surrounding water by adult cladoceran *Daphnia* was larger than that by juvenile individuals, it might have affected the bacteria associated with *Moina* sp. However, the squared distance among the clusters was short (less than 140), and there were some exceptions in the clustering (the 8- and 10-w samples from the NPK+rice straw treatment in the first cluster and the 3-w sample from the NPK treatment in the third cluster). In addition, principal component analysis of the DGGE band patterns did not identify the specific DGGE bands that characterized the respective clusters shown in Fig. 3 (data not shown). These results indicate that the effect of organic matter application and the aging of *Moina* sp. on the eubacterial communities associated with *Moina* sp. in the floodwater was not

appreciable, although Niswati et al. (2003) reported a more distinct effect of the application of rice straw and compost on the eubacterial communities associated with *Moina* sp. by using the PCR-RFLP method. Better resolution of the PCR-RFLP method than that of the PCR-DGGE method may be attributed to the difference in the method of analysis and the targeted length of 16S rDNA: the former between 27 and 1401 positions and the latter between 357 and 517 positions. It was interesting to note that not only eubacterial communities but also dominant eubacteria associated with *Moina* were similar among the treatments, although the application of organic materials seemed to have induced considerable changes in the growth environment for the associated eubacteria.

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